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Teratocarcinoma: neoplastic lessons about normal embryogenesis

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ABSTRACT Germ cell tumors of the testis and the ovary have been studied extensively in humans and experimental animals. Murine teratocarcinomas proved to be one of the best experimental models for elucidating the histogenesis of these tumors and the nature of their undifferentiated stem cells. These spontaneous and experimentally induced tumors, especially those produced from early postimplantation stage embryos, provided a wealth of data about the differentiation of tumor stem cells and the regulation of their growth. This made it possible to draw parallels between the teratocarcinoma cells and their normal equivalents in the embryo. Cumulative data indicate that neoplastic development of murine embryonic cells is just one of the possible ontogenic pathways these cells can take while proliferating in various developmental fields. The malignancy of teratocarcinoma stem cells is determined genetically but can be regulated epigenetically. Development of stem cells in murine teratocarcinomas parallels events in the normal embryo, suggesting that events in the tumor have their normal regulatory counterparts in the embryo proper. The study of early embryos has provided data relevant for oncology, while the study of murine teratocarcinoma helped elucidate some basic developmental events occurring normally in the embryo.

KEY WORDS: *teratocarcinoma, embryogenesis, embryonal carcinoma, yolk sac carcinoma*

Modern teratoma research can be traced back to the pioneering efforts of two scientists: Leroy C. Stevens and G. Barry Pierce. Stevens, working in the Jackson laboratory, Bar Harbor, Maine, noted that 1% of all strain 129 mice develop testicular teratomas (Stevens and Little, 1954). Subsequent studies showed that these tumors originate prenatally from primordial germ cells, which are activated in a way similar to parthenogenetic activation of oocytes (Stevens and Varnum, 1974). Pierce *et al.* (1967) compared ultrastructurally the nascent tumors with other testicular cells and provided additional evidence that the tumors are indeed of germ cell origin.

In addition to benign teratomas 129 mice develop malignant tumors which were appropriately labeled teratocarcinomas (Stevens, 1967). Teratocarcinomas contain malignant stem cells, which accounts for the fact that such tumors may be transplanted to syngeneic animals and thus propagated indefinitely. The stem cells of teratocarcinomas resemble embryonic cells and since they are malignant it is customary to call them embryonal carcinoma (EC) cells.

Since those early days of teratoma research it has been repeatedly shown that EC cells are indeed embryonic and that they resemble normal cells in the early embryos (reviewed by Damjanov and Solter, 1974; Solter and Damjanov, 1979b; Martin, 1980). EC were successfully grown *in vitro* (Bernstine *et al.*, 1973) and numerous cell lines were established (listed in Silver *et al.*, 1983).

Experimentally induced teratomas

The relative scarcity of spontaneous testicular teratomas made it impractical to study the histogenesis of these tumors in 129 mice. Stevens (1973) introduced new genes into the original 129 strain and produced 129/ter-Sv mice that have an incidence of spontaneous tumors in the range of approximately 30 percent. However, few of these tumors were malignant. Thus, spontaneous teratomas are unsuitable for the study of embryonal carcinoma and other malignant stem cells found only in teratocarcinomas.

Our contribution to teratoma research began with the discovery that malignant teratomas, i.e. teratocarcinomas, can be produced from normal embryos transplanted to extrauterine sites of adult syngeneic recipients — such as the space underneath the kidney capsule (Solter *et al.*, 1970). Stevens (1970) transplanted embryos into the testis and obtained similar results. These experiments confirmed beyond any doubt the notion that the stem cells of teratocarcinomas are embryonic and more closely related to normal embryonic cells than germ cells. This also confirmed the hypothesis, championed by Pierce (1967), that teratomas and teratocarcinomas represent caricatures of normal embryogenesis.

Abbreviations used in this paper: EC, embryonal carcinoma.

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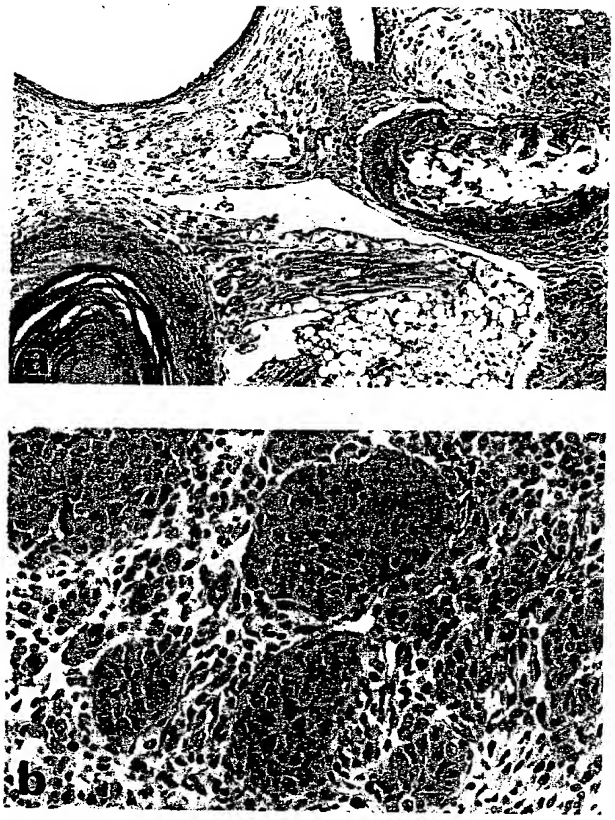

Fig. 1. Histology of embryo of derived teratoid tumors of the mou
(a) *Benign teratoma composed of well differentiated somatic tis*
Teratocarcinoma contains groups of undifferentiated stem cel
embryonal carcinoma cells. **(c)** *Higher magnification of EC cells. Magn*
cations: a, b x240; c x380.

Skreb *et al.* (1971) used early postimplantation stage rat embryos to study the developmental potential of the primordial germ layers which form the embryonic shield at this stage of development. Rat embryos transplanted beneath the kidney capsule of syngeneic adult recipients form teratomas composed of various tissues intermixed haphazardly. Mouse embryos of equivalent age, i.e. 6-7 days *post coitum*, transplanted in an identical manner also give rise to teratomas (Fig. 1a). However, in C3H mice at least 50% of all grafts develop into large tumors that grow progressively and finally kill the host. Such tumors, which are obviously malignant, contain not only teratomatous components but also undifferentiated stem cells that are indistinguishable from EC in spontaneous teratocarcinomas of the testis (Fig. 1b and c).

EC, the stem cells of embryo-derived teratocarcinomas, have many features in common with the embryonic cells from which they have been derived. Most notably, EC are ultrastructurally indistinguishable from the undifferentiated cells forming the inner-most layer of the egg cylinder — known as ectoderm or epiblast (Skreb *et al.*, 1991). Like the epiblastic cells, EC cells are rich in alkaline phosphatase (Damjanov *et al.*, 1971), and express the cell surface carbohydrate antigen known as stage-specific embryonic antigen one (SSEA-1) (Fox *et al.*, 1981). Developmentally, EC are pluripotent, like the epiblastic cells, and can give rise to ectodermal, endodermal and mesodermal structures. Thus EC are either direct descendants of the epiblastic cells in the egg cylinder or equivalent to the normal embryonic cells.

Diwan and Stevens (1976) separated the egg cylinder into constituent components and showed that teratocarcinomas can produced only from the epiblast. Teratocarcinomas can be induced from younger embryos as well (Stevens, 1968), but not embryos that are older than 8 days (Damjanov *et al.*, 1971b). upon transplantation to extrauterine sites give rise exclusively benign teratomas.

On the basis of these experiments we have suggested that represent descendants of normal cells forming the inner mass

TABLE 1

EMBRYO DERIVED TERATOCARCINOMA PERMISSIVE AND NON-PERMISSIVE MOUSE STRAINS

Permissive strains	C3H BALB/c DAB/2
Non-permissive strains	C57BL/6 129 AKR

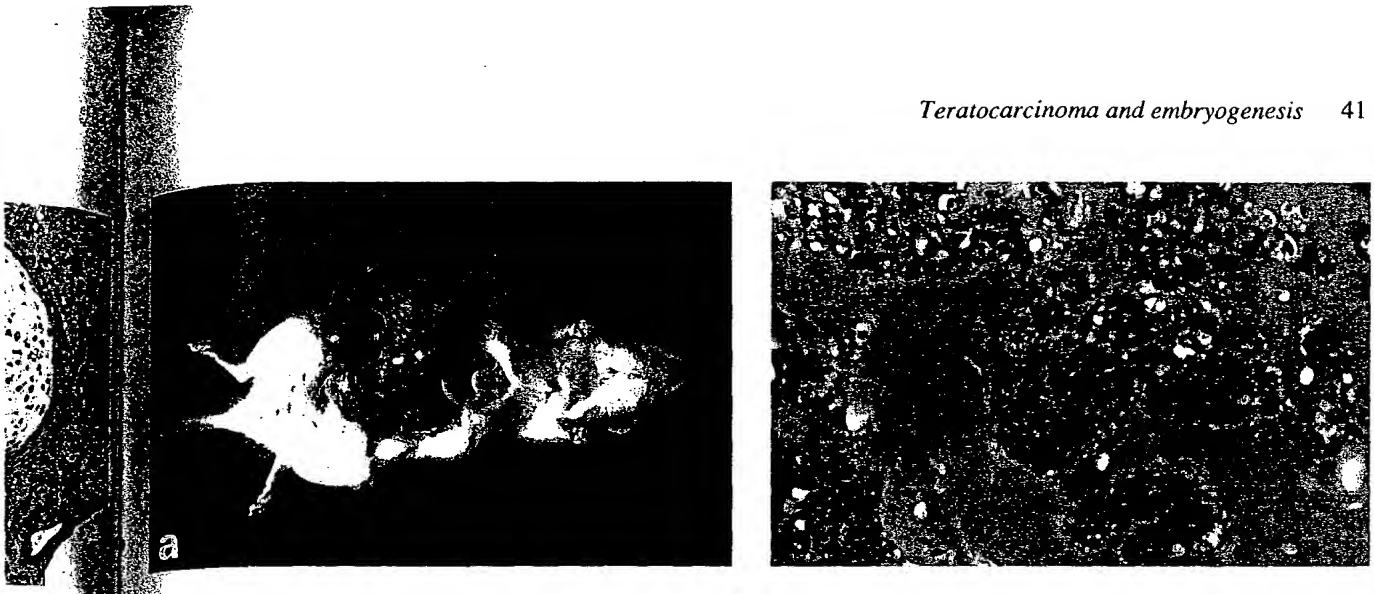

Fig. 2. Embryo derived tumor of the rat. (a) *Gross appearance of a tumor produced by transplanting of 8-day-old rat egg cylinder underneath the kidney capsule of a syngeneic adult Lewis rat.* **(b)** *Histologically these tumors have the appearance of yolk sac carcinomas. Magnification x320.*

the blastocyst or the epiblast of the egg cylinder. These undifferentiated normal embryonic cells transplanted to the extrauterine sites retain their embryonic nature and continue proliferating indefinitely because the adult host does not have the means to control their growth (Damjanov and Solter, 1974). Alternatively, it is also possible that the adult host secretes certain substances that either stimulate the proliferation of embryonic cells, or prevent their differentiation into non-proliferating somatic tissues. The recent discovery that the leukemia inhibitory factor (LIF) stimulates the proliferation of inner cell mass cells of the blastocyst and inhibits their differentiation (Moreau *et al.*, 1988) favors the second hypothesis. In any case, for the time being no factors were identified that favor or inhibit the proliferation of undifferentiated embryonic cells transplanted into the adult host (Damjanov, 1991).

Genetic determinants of teratocarcinogenesis

In contrast to mouse embryos, which give rise to teratocarcinomas upon transplantation to extrauterine sites, rat (Svajger *et al.*, 1986) or hamster (Damjanov, 1978) embryos of equivalent developmental age do not give rise to malignant tumors and form only teratomas. Apparently, the capacity to form embryo-derived teratocarcinomas is a species-specific feature limited to mice. However, even in mice not all inbred strains give rise to teratocarcinomas at the same rate as originally noted for the C3H mouse (Solter *et al.*, 1970). We have thus divided mouse strains into two groups: embryo-derived teratocarcinoma permissive and teratocarcinoma non-permissive strains (Damjanov *et al.*, 1983). Strains like C3H and BALB/c, which give rise to embryo-derived teratocarcinomas in 50% or more grafts, were considered permissive and those that formed teratocarcinomas in fewer than 15% of grafts were classified as non-permissive (Table 1). This shows that the genetic constitution of mice used for transplantation is an important determinant of malignancy in this tumor system.

For reasons that are not fully understood, rat egg-cylinders transplanted to extrauterine sites form only teratomas and yolk sac carcinomas (Fig. 2) (Damjanov *et al.*, 1977). Morphologically yolk sac carcinomas of the rat resemble those described in mice (Pierce *et al.*, 1962).

Epigenetic determinants of teratocarcinogenesis

In an attempt to determine whether the primary determinants of permissiveness or non-permissiveness to embryo-derived teratocarcinogenesis reside in the transplanted embryo or the graft-bearing host, we transplanted 7-day embryos of permissive and non-permissive strain mice into F_1 hybrids produced by crossing the males of non-permissive strains with females of permissive strains and vice versa. In these experiments we found out that the hybrid hosts may abrogate the non-permissiveness of the embryos (Solter *et al.*, 1981). For example, when the C57BL/6 embryos were transplanted to syngeneic hosts, only 6 to 8 percent of grafts gave rise to teratocarcinomas. On the other hand, the same embryos

TABLE 2

MARKERS OF EPIBLAST AND YOLK SAC CELLS OF PERI-IMPLANTATION MOUSE EMBRYOS

		Yolk sac	
	Epiblast (Ectoderm)	Visceral yolk sac	Parietal yolk sac
SSEA-1	+	+	-
SSEA-3	-	+	-
AFP	-	+	-
Keratin	-	+	+
Fibronectin	-	+	+
Laminin	+/-	+	+
Alkaline phosphatase	+	-	-
Acid phosphatase	-	+	-

SSEA= stage specific embryonic antigen defined by monoclonal antibodies; AFP - alpha fetoprotein.
Based on Adamson *et al.* (1985) and Damjanov *et al.* (1990).

Fig. 3. F9 teratocarcinoma cell line. *The cells appear closely related one to another evidenced by the molding of their contours. There are prominent mitochondria in the well developed cytoplasm. Euchromatin predominates in the nuclei. Magnification x4700.*

transplanted to (C57BL/6xC3H) F_1 or (C3HxC57BL/6) F_1 hybrid hosts formed teratocarcinomas in 26 to 46 percent of grafts. It became apparent that all mouse embryos are capable of producing embryo-derived teratocarcinomas but that the outcome of the transplantation depends on the host. It is not clear whether the hybrid hosts actively promote the formation of teratocarcinomas or whether they simply lack the inhibitory influences that operate in inbred teratocarcinoma non-permissive strains. The first alternative seems to be more plausible. It was shown that F_1 hybrid embryos transplanted to teratocarcinoma permissive strains produce larger tumors upon transplantation to F_1 hosts than to syngeneic hosts (Solter *et al.*, 1981), which suggests that the hybrids stimulate the growth of embryonic cells. However not all F_1 hybrids have such a stimulatory effect. Hybrid inhibition of teratocarcinogenesis was also noticed, especially when the embryos of teratocarcinoma-permissive strains C3H and BALB/c were transplanted to F_1 hybrids produced from these two strains. It is of interest to note that these epigenetic factors operating in the adult host appeared maternally linked, which could be explained by imprinting of some genes involved in this process (Damjanov *et al.*, 1983).

The maternal determinants of teratocarcinogenesis operate not only in the adult F_1 hybrid recipients of embryonic grafts but also in the embryo itself (Damjanov and Solter, 1982). The maternal influences operating in the embryo were demonstrated in reciprocal F_1 hybrid embryos produced from teratocarcinoma permissive C3H and teratocarcinoma nonpermissive C57BL/6 parents. We have shown that the F_1 embryos have a high teratocarcinogenic potential if the mother of the embryo is of the teratocarcinoma permissive strain. The potential to form malignant tumors was much lower in F_1 embryos whose mothers were of the teratocarcinoma non-permissive strain. The nature of this maternal effect remains unclear, but could be related to imprinting of maternal genes (Solter, 1988), cytoplasmic factors operating in the cytoplasm of the ovum or the uterine influences. Irrespective of the final explanation of these data, these experiments show that the malignancy of embryo-derived teratocarcinoma depends on both genetic and epigenetic factors.

Immune factors regulating teratocarcinogenesis

In order to test the hypothesis that teratocarcinomas do not develop from embryos that evoke an adverse growth inhibitory immune response of the grafted host, we transplanted 7-day embryos of teratocarcinoma permissive and non-permissive strains to immunosuppressed hosts or nude mice lacking T-cell immunity (Solter and Damjanov, 1979a; Damjanov *et al.*, 1982). It is of interest to note that we could not demonstrate any inhibitory influences of the immune system.

In contrast to our expectations, we found that the teratocarcinoma permissive strain embryos did not grow so well in immunosuppressed animals and concluded that the intact immune system seems to foster teratocarcinogenesis in this tumor system. Apparently the immune system of intact animals secretes some factors that promote the proliferation of undifferentiated embryonic cells, but the nature of this humoral factor remains enigmatic.

The murine early embryonic cells, like the EC cells, do not express the major histocompatibility locus antigen H-2 (Ozato *et al.*, 1985). Indeed some teratocarcinoma cell lines can be grown as ascites tumors in outbred mice (Damjanov *et al.*, 1985). In view of these facts we were surprised to find that teratocarcinomas cannot be produced from 7-day embryos transplanted to non-syngeneic hosts. Apparently the grafts evoke an immune reaction and are destroyed 10 to 15 days after the transplantation.

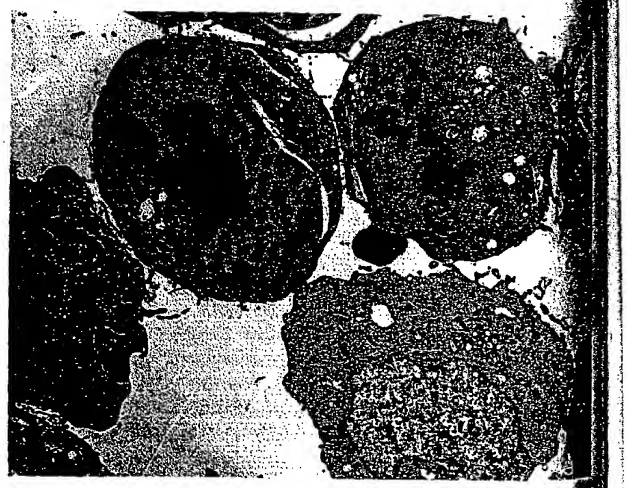

Fig. 4. F9 teratocarcinoma cell line. *The cells are rounded and have surface microvilli. The cytoplasm is filled with free ribosomes and few other organelles. Heterochromatin is prominent in the nucleus. Magnification x4700.*

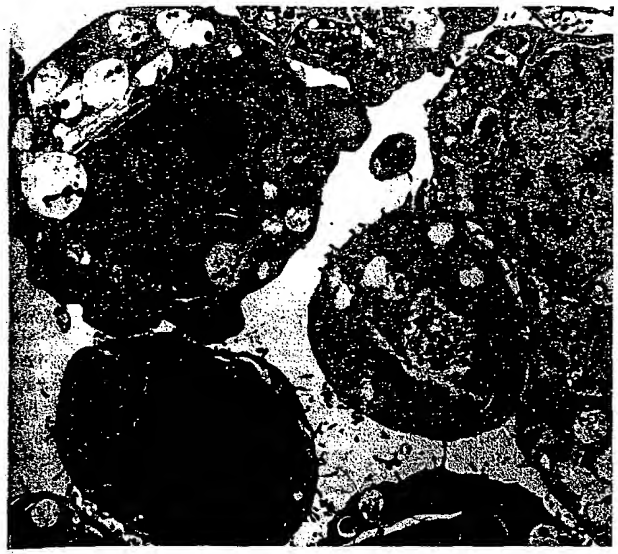

Fig. 5. F9 cells treated with 10^{-6} retinoic acid for 5 days. *Vacuolated cells corresponding to visceral yolk sac, and rough endoplasmic reticulum-rich cells corresponding to parietal yolk sac cells are seen. Magnification x4700.*

We were not able to produce teratocarcinomas from outbred Swiss Webster embryos transplanted to random-bred Swiss Webster hosts. All embryos transplanted to outbred hosts were destroyed by the immune reaction that they elicited. We even tried to transplant embryos underneath the renal subcapsular space of their own mothers but were unable to produce tumors. We also tried to produce limited inbred strains of Swiss Webster mice by sister-brother matings for seven generations. However, this inbreeding was still not sufficient to produce embryo-host compatibility. None of the embryos from these mice survived long enough in the grafted hosts to produce teratocarcinomas. A few long-term surviving grafts were histologically identified as benign teratomas or tumors of the yolk sac phenotype. None of these tumors could be further propagated by heterotransplantation or passaged *in vitro*. One could conclude that the immune factors may inhibit the proliferation of embryonic cells, but the mechanism of this immune reaction remains poorly understood.

Teratocarcinoma derived cell lines

Stem cells of teratocarcinomas are readily passaged from one animal to another and can be cultured relatively easily *in vitro*. A good list of available teratocarcinoma derived cell lines may be found in the appendix of the book edited by Silver *et al.* (1983). Many of these cell lines have been fully characterized but many others remain incompletely defined. It is of interest to note that the nature of some cell lines, even though they are widely used, remains controversial. Suffice it to say in this context that the most widely used teratocarcinoma derived cell line, F9 (Bernstine *et al.*, 1973), represents to some scientists a typical embryonal carcinoma; to others it is a developmentally committed embryonal carcinoma, while to still others it is a primitive endoderm-like cell line. It was initially thought to be developmentally multipotent, until Sherman and Miller (1978) showed that it differentiates spontaneously into yolk sac cells. Subsequently it was shown that the differentiation of F9 cells can proceed into visceral or parietal endoderm (yolk sac) depending on the culture conditions (Hogan *et al.*, 1983). Other forms of differentiation, such as neural cell formation reported by some investigators could not be confirmed (Tienari *et al.*, 1987). The issue of the developmental potential of F9 cells has however not been fully resolved, and it appears that somatic cell derivatives can be grown from these cells transfected with oncogenic viruses (Kellerman *et al.*, 1990).

We have studied F9 cells from different sources and have noticed minor, but possibly important, differences. In one cell line that we acquired as F9 from Dr. L. Grabel (Grabel and Casanova, 1986), there is obvious close juxtaposition of cells which tend to form monolayers (Fig. 3). These cells have moderately developed cytoplasm full of free ribosomes and scattered mitochondria. The nuclei vary in size and shape but contain, in addition to a well developed nucleolus, mostly euchromatin. The cells of another F9, acquired from Dr. D. Solter, grow without prominent intercellular contacts (Fig. 4). The cell surface of these cells projects into short microvilli. Their nuclei contain heterochromatin granules interspersed with euchromatin through the entire nucleus or attached to the nuclear membrane. The cytoplasm of these cells is also filled with numerous free ribosomes but contains fewer mitochondria. Scattered short profiles of rough endoplasmic reticulum are also present. On the basis of these electron microscopy data it appears that these two cell lines are different — the first one being more similar to the «idealized» inner cell mass of the blastocyst or epiblast of the egg cylinder, and the second line having features of primitive endoderm. Yet both cell lines respond well to retinoic acid and differentiate within a few days into cells that have features of parietal or visceral yolk sac cells (Fig. 5). Obviously continuous propagation of F9 cells in various laboratories resulted in inadvertent cloning of subsets of cells that now grow as permanent lines, differ one from another, but are still called F9.

We have studied several teratocarcinoma cell lines established in our laboratory or elsewhere (Fox *et al.*, 1983; Damjanov *et al.*, 1990), and compared them with human equivalents (Andrews *et al.*, 1987; Damjanov, 1990). As a rule these cells tend to be rather susceptible to culture conditions and tend to change their morphology depending on the culture medium, use of feeder layer, or gelatin coating of culture dishes. Even after several cloning attempts many cells lines tend to be pleomorphic. Some of this pleomorphism probably reflects the tendency of teratocarcinoma stem cells to differentiate spontaneously into other cell forms and in part it reflects the changes induced by growth conditions. For example the parietal yolk sac carcinoma cell line ME (Damjanov *et al.*, 1990) forms flattened cells, rich in rough endoplasmic reticulum attached to the surface of the plastic dishes. The cells that detach from the surface and float in the medium tend to vacuolate and transform into balloons filled with fluid.

Using electron microscopy, immunohistochemistry and by assessing the development potential or the secretory activity of various cell lines, we were able to identify cell lines as corresponding to distinct cell populations in the mouse embryo from the blastocyst to the primitive streak stage of development. Embryonal carcinoma cell lines, like NE (Damjanov *et al.*, 1990) or NF-1 (Fox *et al.*, 1983) are truly primitive cells showing no signs of cytoplasmic differentiation, and in essence resemble F9 cells illustrated in Fig. 3. A cell line that we have established *in vitro* from the ascites tumor

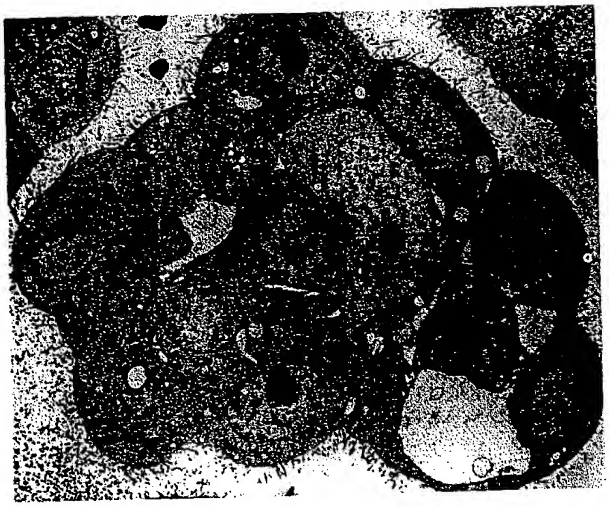

Fig. 6. Tumor cell line C44 forms embryoid bodies composed of an inner cell mass of embryonal carcinoma cells and outer yolk sac-like cells. *Magnification x3500.*

C44 provided by Parchment *et al.* (1990) consists of similar cells which, however, spontaneously differentiate into yolk sac cells forming with them embryoid bodies (Fig. 6). It is notable that these EC cells in the inner mass of embryoid bodies also form prominent intercellular junctions.

The cell line Ter-C (Searls and Edidin, 1982) corresponds to primitive endoderm. Typically, this cell line has more heterochromatin in the nucleus and contains more rough endoplasmic reticulum in the cytoplasm than the typical undifferentiated EC cells (Fig. 7). In contrast to parietal yolk sac carcinoma cells like the aforementioned ME cell line, Ter-C cells have narrow profiles of rough endoplasmic reticulum and produce small amounts of basement membrane material and plasminogen activator. In contrast to typical EC cells, Ter-C cells do not express the stage-specific embryonic antigen one (SSEA-1) and have no alkaline phosphatase activity on their cell membrane.

Yolk sac carcinoma cell lines that we have examined were either pure parietal yolk sac carcinomas, like the cell line ME, or a mixture of parietal and visceral yolk sac cells, like the cell line LRD (Damjanov *et al.*, 1990). The parietal yolk sac cells are characterized by the abundance of rough endoplasmic reticulum, which contains basement membrane-like material secreted by these cells (Wewer *et al.*, 1987). These cells do not express alkaline phosphatase, nor the carbohydrate markers SSEA-1 and SSEA-3, which are found on EC cells and visceral yolk sac cells, respectively. Parieto-visceral cells, like the cell line LRD, tend to be polarized and form aggregates which on their free surface display prominent long microvilli. These cells have long intercellular junctions, contain frequent cytoplasmic vacuoles, and tend to show marked pinocytotic activity, like the normal visceral yolk sac cells in the midgestational embryo (Jollie, 1990). Additional features distinguishing various forms of yolk sac cell lines from each other were studied by Adamson *et al.* (1985) and have been summarized in our recent paper (Damjanov *et al.*, 1990), and are listed in Table 2.

D v lopmental biology of terat carcin ma st m cells

As predicted by Pierce (1967), teratomas consist of cells th have their equivalents in the normal embryo. Pierce *et al.* (19 have shown that the parietal yolk sac carcinoma correspond cells lining the Reichert's membrane of the parietal yolk sac in conceptus. The studies of several other laboratories, includi ours, have extended these original observations to other cell have documented fully the validity of this concept (Pierce Speers, 1988). New cell lines are still being developed, markers are becoming available. We believe that it is not far the day when well characterized malignant replicas of cru embryonic cell forms will become available. This will enable u further explore the similarities between normal cells in the em and their malignant counterparts in teratocarcinomas. Also, thi provide sufficient amounts of embryonic cell surrogates for chemical studies. At the same time these cell lines will enable to construct genealogical charts for various tumor stem cell different maturities and levels of differentiation, analogous ontogenetic charts of cell lineages in early embryos (Fig. 8) comparing tumor stem cells and embryos we hope to prove there are several phenotypes of EC or yolk sac carcinoma and th

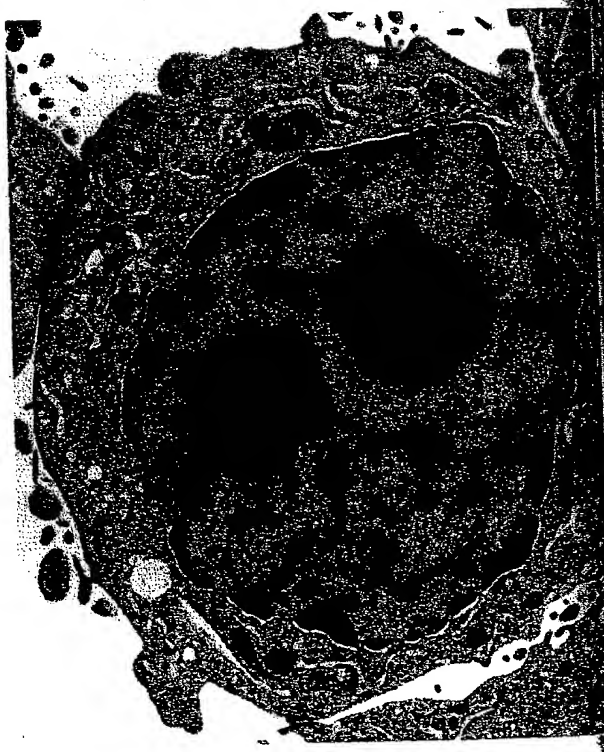

Fig. 7. Cell line Ter-C corresponding to primitive endoderm. *The cel are interconnected with desmosomes. The cytoplasm contains marro profiles of rough endoplasmic reticulum. The nucleus contains promine heterochromatin and nucleoli. Magnification x14000.*

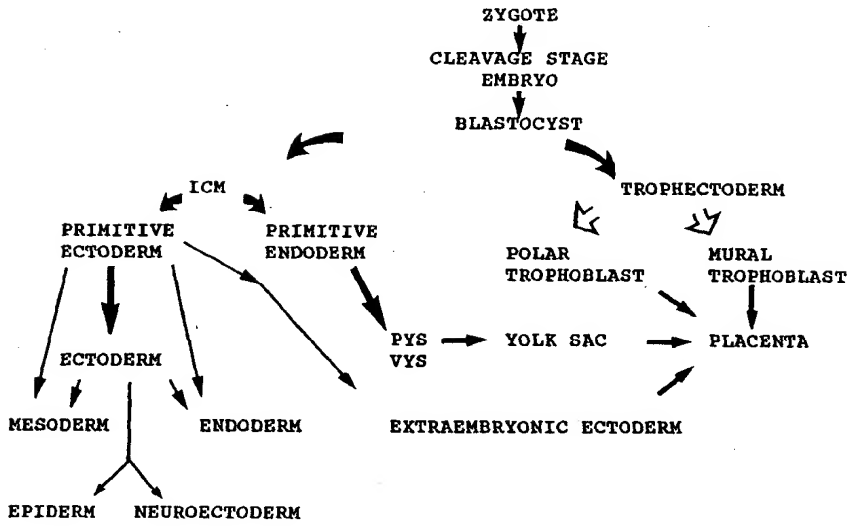

Fig. 8. Histogenetic chart of early murine embryogenesis shows cell lineages and the plasticity of stem cells at various stages of development. *EC cells correspond to the inner cell mass (ICM)-primitive ectoderm (epiblast) cells. Primitive endodermal cells and parietal yolk sac (PYS) carcinoma cells have been isolated, as well as a trophectodermal carcinoma cell line. There are no pure visceral yolk sac (VYS) cells. In due time one could expect that tumor cell lines corresponding to other embryonic cells will be isolated as well.*

these cell types correspond to normal cells at distinct stages of embryogenesis. Heterogeneity of teratocarcinoma stem cells could then be understood in terms of normal embryogenesis, just as many aspects of embryonic development have been clarified by the study of teratocarcinoma.

Acknowledgments

I heard of G. Barry Pierce for the first time in 1969 in New York when I was a resident in pathology. On the bulletin board I saw a pamphlet describing the training program that he had inaugurated as the new Chairman of the Department of Pathology, University of Colorado in Denver. The advertisement intrigued me and although I considered going to visit Prof. Pierce. We did not meet then. I returned to Zagreb to continue my training in pathology and work in the embryology laboratory of Professor Nikola Skreb. In the meantime, Barry must have read some papers that I coauthored with Davor Solter and Professor Skreb. I assume that he read our articles since he recommended us to the organizers of the first Congress of Differentiation in Nice in 1971, the proceedings of which were published the next year (Skreb et al., 1972). I never made it to Nice and met Barry only a few years later, when he site-visited my laboratory in Farmington, Connecticut. As a result of that visit I was awarded my first NIH grant. Barry Pierce enabled me to become an experimental pathologist.

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